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Mothers' Consumption of Soy Drink But Not Black Tea Increases the Flavonoid Content of Term Breast Milk: A Pilot Randomized, Controlled Intervention Study

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Abstract
Objective: We performed a pilot RCT to prove the hypothesis that a controlled ingestion of polyphenol-rich beverages (soy drink, decaffeinated black tea) in nutritive dosages by nursing women has an effect on the composition (flavonoid concentration, total antioxidant capacity) of breast milk. ***Methods:*** Healthy nursing women were supplemented with either 250 mL of a soy drink (12 mg isoflavones; $n = 18$), 300 mL decaffeinated black tea (67 mg catechins; $n = 18$), or 300 mL water ($n = 8$, control) for 6 days. Milk samples were collected before, during, and after intervention. Flavonoid content (isoflavones/catechins, HPLC) and total antioxidant capacity of milk and test drinks in milk specimens were assessed. ***Results:*** Isoflavone content (genistein and daidzein) in breast milk increased up to 12 nmol/L after soy drink consumption; the major flavonoids constituents of black tea (catechin, epicatechin, and respective conjugates) could not be detected in milk samples. With both interventions, the total antioxidant capacity of breast milk was not affected. ***Conclusions:*** Mothers' daily consumption of a soy drink considerably increases isoflavone content of breast milk resulting in an estimated daily exposure of 9.6 nmol isoflavones in a 4-month-old suckling infant. Luminal flavanol uptake from black tea consumed by the nursing mother may be too low to affect flavanol concentrations in breast milk.

Introduction

A striking outcome of numerous observational studies in adults is that regular consumption of plant-derived foods and drinks (soy milk, black, and green tea, cocoa) in comparably large quantities (500–600 g/day) essentially contributes to mitigating the overall risk of degenerative diseases such as cardiovascular disorders, specific forms of cancer, osteoporosis, or diabetes [1, 2]. These preventive effects are mainly attributed to characteristic polyphenols formed during the "secondary metabolism" of plants. Flavonoids in particular exhibit strong in vitro antioxidant properties that may effectively contribute to counteract oxidative cell or organ damage in vivo [3–5]; additional anti-inflammatory effects may positively support bone and vascular health, and brain functions in adults [6–8].

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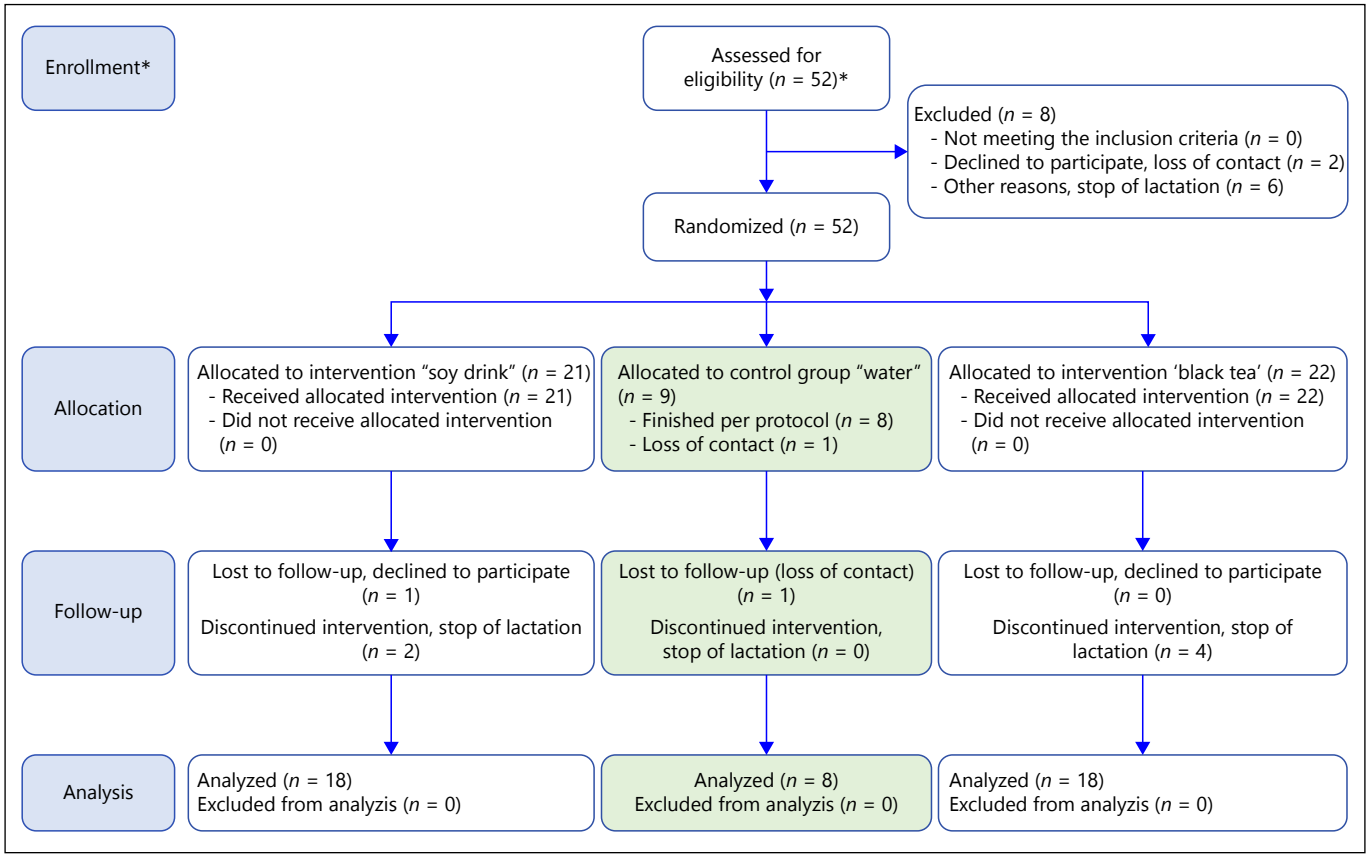

Fig. 1. Flow diagram of the pilot RCT. * The enrollment was continued until $n = 18$ finished the study in every verum group.

Recent analytical studies demonstrated that breast milk samples collected under free-living conditions contain food-derived polyphenols like epicatechin, quercetin, kaempferol, genistein, and daidzein in varying quantities and qualities [9, 10]. The first evidence that polyphenols in the diet of lactating mothers can be found in breast milk was reported by Franke et al. [11]. After one lactating woman consumed 20 g of roasted soybeans, significant amounts of isoflavones could be detected in breast milk samples. In a more recent study from the same group, 18 mothers were prescribed one serving of a soy protein–based beverage (36.5 g; 24.3 mg daidzein, 28.5 mg genistein) over a period of 2–4 days; breast milk samples were collected once on the last day of the intervention [12]. Soy intake increased isoflavone content in breast milk by a factor of 10–15. Controlled intervention studies with the goal to evaluate the transfer of mothers' dietary flavanols into breast milk are limited. In a very recent study, 2 healthy lactating mothers ingested one portion of dark chocolate corresponding to 80 mg of flavanol monomers. In breast milk samples collected within 12 h after ingestion, epicatechin sulfates could be detected only in trace amounts (0.01% of the chocolate-derived flavanol intake) [13].

Only one additional intervention study focusing on plant antioxidants can be found in the literature: ingestion of non-alcoholic beer, a product rich in antioxidants like ubichinons, during early lactation significantly increased TAC in breast milk compared to controls [14].

The aim of our pilot RCT was to prove the hypothesis that mothers' consumption of isoflavone and flavanol-rich food intake during lactation can increase breast milk contents of flavonoids, thereby increasing its antioxidant capacity.

Subjects and Methods

This pilot RCT study was approved by the Ethics Committee of the "*Ärztekammer Berlin*" (Medical Association of Berlin). Enrollment of participants, allocation, follow-up, and analysis procedures are summarized in Figure 1.

Recruitment of Milk Donors

We recruited apparently healthy mothers who had given birth to infants at the *Evangelisches Waldkrankenhaus* Spandau, Berlin (Germany) between 35 and 42 (+6) weeks of gestation and had undergone at least 27 days of unproblematic nursing for study participation (recruiting period: 24 months). Mothers were initially contacted during their post-childbirth hospital stay. Between days 15 and 27 of lactation, mothers interested in study participation were visited at home in order to provide them with information on study details and on exclusion criteria (drug use, consumption of nutritional supplements, acute/chronic metabolic diseases, smoking, BMI <15 or >30 kg/m^2, allergies, specific diets, eating disorders, or participation in other studies). The mothers were then requested to provide their written consent to participate in the study. After confirmation, the standardized nutritional 3d-food questionnaire that focused on energy, antioxidant, and flavonoid intake was explained.

Study Protocol and Sample Handling

Participants were then randomized (lottery block randomization with unpaired group sizes) in one of the 3 study groups; test beverages were provided after randomization. Group I was advised to consume one daily serving (250 mL) of a soy drink (Alpro, Düsseldorf, Germany), Group II consumed 2 cups (300 mL) of decaffeinated black tea (3 g Darjeeling per brewing; TeeGschwendner, Meckenheim, Germany), and Group III drank 300 mL of water to control for diet-dependent changes in plasma polyphenol concentrations.

All participants were advised to consume the study drinks in the morning over a period of 6 days in addition to their "normal" diet. The mothers were instructed to avoid consumption of flavonoid-rich beverages or foods (besides the test drinks) during the study period (e.g., tea, coffee, soy-based food, or cocoa-containing food). Nutrition intake was assessed prior to and during the first 2 intervention days using a standardized 3-day food record. Milk samples (>10 mL) were taken at before intervention (day 1), during intervention (days 4 and 7), and after intervention (day 8) 5 min after start of a given breastfeeding interval in the evening (about 10 h after study drink ingestion) using a breast pump; samples were immediately cooled.

The samples were initially stored for maximal 14 days in the household freezer (–18°C), subsequently transported on dry ice to the Department of Nutrition and Food Sciences in Bonn, Germany and stored at –80°C until analysis. Prior to analysis, samples were centrifuged (1,800 *g*, 5 min) in order to remove milk fat.

All measurements were performed in duplicate, with the mean value used for calculations. The laboratory staff was blinded with respect to the origin of the samples.

Analytics

Isoflavones

Soy drink samples were treated with glucosidase; isoflavone aglycones were subsequently extracted according to the method by Knight et al. [15] using methanol. Dried extracts were stored at –80°C until analysis. Skimmed breast milk specimens (1 mL) were mixed with 50 µL of an aqueous glucuronidase/sulfatase enzyme mixture (200 kU/L; Sigma Aldrich, Taufkirchen, Germany) and 250 µL acetate buffer (pH 4; final pH in the sample approx. 5) prior to 1-hour incubation in a water bath (38°C) for complete deconjugation of sulfatated and glucuronated isoflavones. Isoflavones (daidzein and genistein) were subsequently extracted via solid-phase extraction (SPE) by means of polymer-based Strata X cartridges (Phenomenex, Aschaffenburg, Germany) and 2 cycles of methanol elution [16]. The recovery rates represented 97 ± 4% (daidzein) and 104 ± 2% (genistein) respectively. Dried extracts were stored at –80°C until analysis.

High-performance liquid chromatography (HPLC; Sykam, Munich, Germany) of pretreated soy drink and milk specimens was performed on reversed-phase material (ProntoSIL C18H, 250 × 4.6 mm, 120Å, 5 µm; VDS optilab, Berlin, Germany), and subsequent electrochemical detection (EP 30, Biometra, Göttingen, Germany; potential 750 mV) was carried out. Isocratic elution (0.9 mL/min) was conducted with ammonium acetate buffer (pH 4.6) methanol/EDTA (50/50/1 v/v). The precision (closeness of agreement among a set of results) of the method was validated by repeated analysis (n = 10) of the same standard solution: the coefficients of variation were 4.2 and 3.3% for daidzein and genistein respectively. By using the signal-to-noise (3:1) method (noise magnitude measured manually on the chromatogram printout) [17], a limit of detection (LOD) of 6.25 nmol/L (daidzein) and 2.5 nmol/L (genistein), respectively, could be estimated. Detector response was linear (r^2 > 0.99) within the range from 5 to 80 nmol/injection (injection volume 20 µL).

Flavanols

Tea infusions (3 g decaffeinated Darjeeling, stewed in boiling water for 3 min) were diluted (1:10) with water. A specimen of 1 mL was subsequently mixed with 3 mL methanol (containing 1g BHT/L to avoid oxidation) for 5 min. Flavanol extraction was then performed according to Kivits et al. [18] using aluminum oxide. Skimmed breast milk specimens were mixed with 250 µL 0.1 M sodium phosphate buffer (pH 3.5 including 0.5 mM Na2-EDTA) and incubated (1 h, water bath at 37°C) using an aqueous solution of glucuronidase/sulfatase (200 kU/L; Sigma Aldrich, Taufkirchen, Germany). After adding ethyl gallate (10 µmol/L as internal standard), flavanols (catechin [Cat], epicatechin [Epi], epigallocatechin gallate [EGCG], epicatechin gallate [ECG]) were extracted using SPE (hydrophilic-lipophilic-balanced, reversed-phase cartridge; OASIS®HLB, Waters, Eschborn, Germany) according to Unno et al. [19]. Recovery for Cat was 101 ± 1% and for Epi 107 ± 2%. The extracts were evaporated and stored at –80°C until analysis.

HPLC (Sykam, Munich, Germany) was performed on reversed-phase material (ODS Hypersil, 250 × 4.6 mm; Thermo Electron Corporation, Waltham, USA), with subsequent electrochemical detection (EP 30, Biometra, Göttingen, Germany; potential 650 mV). Isocratic elution (0.6 mL/min) was carried out using 0.2 M phosphoric acid/acetonitrile/tetrahydrofuran (86/12.5/1.5 v/v). The precision of the method was validated by repeated analysis (n = 10) of the same standard solution: CVs for flavanols were 4.7–8.0%. LOD at a signal-to-noise ratio of 3:1 [17] were 2.5 nmol/L (Cat; Epi) and 5.0 nmol/L (EGCG and ECG) respectively. Detector response was linear (r^2 > 0.99) within the range from 5 to 80 nmol/injection (injection volume 20 µL).

Antioxidant Capacity

TAC was analyzed by means of the ATBS assay [20, 21]. In brief, skimmed breast milk samples were mixed with hexane (1:1 v/v) and shaken vigorously. After centrifugation (1,800 *g* for

10 min), lipophilic and aqueous phases were collected separately. Prior to analysis, dried samples were dissolved in 200 µL PBS (5 mM, pH 7.4), placed in a cuvette, and mixed with metmyoglobin (70 µmol/L) and ABTS (500 µmol/L). Reduction in ABTS molecules was traced spectrophotometrically at 734 nm and expressed as total, lipophilic, and hydrophilic antioxidant capacity in mmol/trolox equivalent (mmol/L TE).

Data Analysis

Dietary intake in terms of energy, vitamins A, E, and C, as well as zinc was calculated based on the 3-day food records using the software EBISpro® (Hohenheim, Germany) in conjunction with the German database of nutritional values for foods, (Bundeslebensmittelschlüssel, BLS II.3). Dietary isoflavone and flavanol intake were calculated using USDA databases and recent food analyses [22].

All values were determined in duplicate and the mean values were calculated. In the event that significant discrepancies arose between the 2 measurements, both measurements were repeated.

Results

Within the predefined recruitment period, 49 mothers provided their written consent to participate in the study; 5 did not complete the study protocol for various personal reasons (e.g., insufficient breast milk production, non-compliance to study protocol, loss of contact). Anthropometric data of the final 44 participants are summarized in Table 1; characteristics did not differ between groups.

Dietary energy, vitamin C, vitamin E, zinc, and flavonoid intakes (3d-food record) were comparable in all groups (Table 2).

Considering the flavonoid content of the 2 test drinks used for the intervention (Table 3), soy drink consumption increased the average daily isoflavone (daidzein plus genistein) intake from 0.2 mg (baseline; Table 2) to 12.7 mg; with black tea daily total Cat, Epi, EGCG, and ECG intake increased from 11.5 mg (baseline; Table 2) to 99.0 mg during the intervention period.

In all milk samples collected prior to, during, and after black tea consumption, flavanol concentrations were below the LOD (data not shown).

In milk samples collected during black tea or water consumption, genistein and daidzein were not detectable (data not shown). When the soy drink was consumed, both daidzein and genistein were detectable in stable concentrations during the intervention; on day 8, concentrations decreased only slightly (Table 4).

TAC constantly reached 1.3–1.4 mmol TE/L with a lipophilic component of 0.1 mmol TE/L without any intervention and/or time effects.

Table 1. Age and anthropometric data of participants in the 3 study groups

	Group I (n = 18)	Group II (n = 18)	Group III (n = 8)
Age, years			
Mean ± SD	31±7	31±4	29±2
Min/max	20/43	25/40	26/31
Weight, kg			
Mean ± SD	68±10	70±10	74±8
Min/max	53/85	53/90	61/86
Height, cm			
Mean ± SD	170±5	168±8	169±7
Min/max	162/180	150/180	156/175
BMI, kg/m^2			
Mean ± SD	24±4	25±3	26±2
Min/max	19/30	20/30	23/28

n, number of individuals in the investigated group; BMI, body mass index.

Discussion

Our exploratory pilot RCT in lactating women was designed on the hypothesis that ingesting black tea or a soy-based test drink increases the polyphenol content of breast milk. The average BMI of participants lay in the upper normal range (Table 1), while self-reported energy intake (Table 1) amounted to 75% of current German recommendations for nursing women [23, 24], thus normally sufficient in covering essential nutritional needs. Since the participants were asked to avoid consumption of polyphenol-rich food items during the study period, dietary intake of isoflavones and flavanols (Table 2) was lower than previously reported for middle-aged Europeans. Based on FFQ data from the EPIC study, Boker et al. [25] calculated a mean daily intake (arithmetic means) of 0.37 ± 1.24 mg for daidzein and 0.42 ± 1.33 mg for genistein in Dutch women (age: 57.1 ± 6.0 years). The total nutritional intake of Epi, ECG, EGCG, and Cat reached 21 mg/day in our study population; only about 1/3 of the intake of these flavanol subgroups reported for adults (men and women) living in Central Europe (72 mg/day) [26]. The timing of daily milk sampling (10 h after drink consumption) considered both available data on the kinetics of luminal uptake and blood levels of polyphenols after oral intake (half-life times between 2.5 and 8.5 h) [13, 27–29] and physiological pathway of milk secretion. Supplemental amounts of isoflavones and flavanols (Table 3) were within an upper physiological range and comparable with doses administered in previous intervention studies in adults (e.g., 300–500 mL green tea corresponding to 200–600 mg flavanol

Table 2. Daily nutritional intake (mean ± SEM) of energy and antioxidants (calculated on the basis of a 3d-food record)

	Group I tea (n = 18)	Group II soy drink (n = 18)	Group III water (n = 8)	Mean all groups (n = 44)
Energy, kcal/day	2,117±167	2,346±122	2,411±323	2,264±102
Vitamin C, mg/day	143±20	164±27	102±28	145±15
Vitamin E, mg/day	12±1.2	13±1.2	12±3.2	12±0.9
Zinc, mg/day	12±0.7	13±1.2	12±1.1	12±0.6
Daidzein, µg/day	142±54	93±64	130±34	119±24
Genistein, µg/day	122±29	140±31	81±18	125±18
Catechin, mg/day	4.2±0.9	15.8±2.4	4.1±1.6	9±3.5
Epicatechin, mg/day	4.9±1.2	8.0±1.8	4.2±1.2	6±0.9
Epicatechingallate, mg/day	0.6±0.1	2.1±0.5	2.9±2.2	1±0.8
Epigallocatechingallate, mg/day	1.8±1.0	7.7±1.8	4.9±3.5	5±2.0

DA, daidzein; GE, genistein; Cat, catechin; Epi, epicatechin; ECG, epicatechin content; EGCG, epigallocatechin gallate; n, number of individuals in the investigated group.

monomers) [11, 12, 30]. The chromatographic methods applied allowed for sensitive and reproducible analysis of isoflavones and flavanols in breast milk samples and test drinks. The study design, thus, enabled reliable measurement of the effects of soy drink and black tea consumption on polyphenol content in breast milk samples.

Before intervention, flavonoids could not be detected in the breast milk samples (Table 3). This result contrasts recent observation by Song et al. [10] analyzing breast milk samples given at several time points from 17 nursing US American women who delivered healthy term infants. ECG and other flavonoids like hesperidin, naringin, and quercetin could be detected in all samples; the aglycone epicatechin, however, could be found only in 8 specimens. Isoflavones were not analyzed. Song et al. [10] used LC-MS technology with LODs in the similar range than calculated for our analytical system. It can, thus, be speculated that the general flavonoid intake of young US American women might be higher than in our subjects. Dietary records to verify this assumption were not available.

Obviously, mothers' black tea consumption did not lead to detectable flavanol concentrations in breast milk (Table 3). The low luminal absorption rates (max. 5% of oral intake) calculated for healthy adults [27–29] may be associated with only a slight increase in plasma flavanol concentrations. If flavonoid transport in the mammary gland follows, as suggested [31] by a paracellular mechanism (direct exchange between blood and milk), considerably higher flavanol blood levels than those observed after consumption of 600 mL black tea might be necessary to effectively secrete flavanols into the lumen of the alveoli. Probably, such concentration may not be reached by oral flavanol uptake.

Table 3. Flavonoid content and antioxidative capacity of the test drinks (duplicates, mean ± SD)

	Black tea, mg/300 mL	Soy drink, mg/250 mL
Daidzein	–	7.6±0.5
Genistein	–	4.8±10.0
Catechin	0.9±0.1	–
Epicatechin	6.3±0.3	–
Epicatechingallate	20.2±1.4	–
Epigallocatechingallate	39.6±4.3	–
	mmol trolox equivalent/L	
Total antioxidative capacity	15.6±1.06	5.0±1.3

Table 4. Isoflavone concentrations (nmol/L, mean ± SD) in human breast milk samples before (day 1), during (days 3 and 6), and after (day 7) intervention with soy drink

	Study day 1	Study day 4	Study day 7	Study day 8
Daidzein	<DL	9.3±4.2	9.1±4.8	7.3±3.3
Genistein	<DL	4.9±7.2	4.3±2.7	3.5±2.3

<DL, below detection limit (daidzein: 6.25 nmol/L; genistin: 2.5 nmol/L).

Mothers' consumption of a soy drink containing a sum of 12 mg genistein and daidzein (a dosage 60-fold higher than dietary intake) increased breast milk concentrations of these solutes (Table 3). In the early intervention study by Franke et al. [11] in one Caucasian woman, an intake of 16.5 mg daidzein and 18.2 mg genistein (about threefold higher doses than those consid-

ered in our study) from roasted beans increased genistein and daidzein concentrations in milk samples up to 30 and 50 nmol/L respectively. Taking both studies together, a linear relationship between mothers' isoflavone intake from soy products and isoflavone secretion in the mammary gland can be hypothesized. Larger intervention studies are now required to confirm this hypothesis.

Assuming a daily average milk volume of 800 mL of a 4-month old infant, our data suggest an isoflavone intake (genistein plus daidzein) of 11 nmol (2.8 μg). Commercial soy-based infant formulas contain 32–47 mg/L (0.12–0.18 mmol/L) total isoflavones, resulting in an about 1,000-fold higher intake compared with breastmilk feeding. In an earlier pilot study analyzing isoflavones in urine, saliva, and blood of infants fed with either soy formula, cow milk, or breast milk, in most of the samples (>90%) from breast or cow milk fed infants daidzein and genistein were undetected, whereas in most of the samples from infants fed with soy formula, these isoflavones were present in comparably high amounts [32]. A recent meta-analysis could not find any negative effects of a high isoflavone intake from soy formulas (but also no benefits) on endocrine and other physiological functions of breastfeeding [33]. The authors speculated that most of isoflavones detectable in the plasma of soy fed infants are present in a conjugated form, which cannot link to isoflavone receptors. Whether the minor amounts of isoflavones present in breast milk after dietary soy intake of lactating mothers may increase free isoflavone plasma concentrations in infants is not known.

Nowadays, TAC is a well-accepted marker to assess food quality. TAC measured in our tea infusions (Table 2) falls within the range of earlier analytical data [34]. The threefold lower antioxidant capacity of soy milk compared to black tea (Table 2) may be explained by its low flavonoid content and antioxidant (pro-)vitamins.

Within the last years, several studies assessed the TAC of breast milk samples [13, 35–41] collected under various physiological conditions (e.g., different stages of lactation, from mothers delivered term or pre-term). Since the analytical methods to analyze TAC slightly differed in the studies performed, a reliable comparison of the data is difficult. It can be, however, concluded that TAC values reported were in a similar range (about 1–2 mmol TE/L). Obviously, our data are within that physiological frame; an influence of supplementation could not be observed. Associations between controlled dietary supplementation of the weaning mother and the TAC in breast milk could be shown only for symbiotic supplementation [42] and non-alcoholic beer [14]. As shown in cross-sectional studies, increased consumption of foods like cheese, vegetables, fruits, bread, and nuts [37] and a comparably high intake of dietary (pro)-vitamins were associated with an increased TAC in breast milk samples [34]. Probably, a health-promoting diet as recommended for lactating mothers including a high proportion of plant-based foods may ensure high TAC in breast milk. Controlled studies to evaluate whether a high TAC of breast milk may beneficially effect TAC of extra-/intracellular compartments of breast feed infants are still lacking.

Clear limitations of our study design concern the analytical methodology and the duration of intervention. We decided to focus on the major components of isoflavones and flavonoids in the text drinks. Other polyphenols (e.g., glycitein, equol, quercetin) were not considered; this may lead to an underestimation of antioxidant polyphenol content in drinks and breast milk samples. For practical reasons, we have chosen a short intervention period; we cannot exclude the fact that a longer supplementation would have shown "cumulative" effects on breast milk polyphenol contents.

In conclusion, our pilot intervention study provides clear evidence that low dietary isoflavone intake of lactating women influences the breast milk isoflavone content. This result supports the notion of a paracellular transport of isoflavones into the alveoli. Due to the generally low luminal uptake of flavonoids, these substrates may not reach the mammary gland in significant concentrations even when high amounts of black or green tea are consumed orally and may explain the absence of flavonoids before intervention. Larger studies are required to confirm these results and to determine whether the levels of breast milk isoflavones have positive or negative effects for the infant.

Disclosure Statement

The authors declare no conflicts of interest.

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